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13. ABSTRACT *(Maximum 200 Words)*

Traditional therapies for advanced prostate cancer are unable to cure a majority of patients. An approach to therapy we have tested involves production of nitric oxide (NO) by introduction of replication defective adenovirus containing the DNA sequences for inducible nitric oxide synthase (iNOS). An adenovirus vector with an iNOS cDNA and a CMV promoter (Ad5-CMV-iNOS) was constructed and tested. The production of nitric oxide in prostate cancer cells after infection with Ad5-CMV-iNOS was measured. Maximum NO release rate occurred when cells were infected at multiplicity of infection (MOI) 15 and after 24 to 36 hours incubation. Direct cell killing of cells growing in monolayer commenced within 1 day after infection and occurred at MOI greater than 5. An *in vitro* cell survival investigation utilized Du-145 and PC-3 prostate tumor cells growing to high density in a collagen matrix. Both control viral vector and Ad5-CMV-iNOS produced direct cell kill but no radiation sensitization in these cell systems. Sensitization was not observed in either aerobic or hypoxic conditions. Sensitization was also not observed after Ad5-CMV-iNOS gene therapy of Du-145 and PC-3 xenograft tumors in nude mice as assayed by the in vivo/in vitro method.

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4) INTRODUCTION

Traditional therapies for advanced prostate cancer are unable to cure a majority of patients. Improving local control of the primary tumor is a practical means of increasing the cure rate. Local failure after radiation therapy may result from treatment resistant hypoxic cells. Preferentially killing or radiation sensitizing hypoxic and/or nutrient deprived cells should improve local response and cure rate. One approach to cytotoxic and sensitization therapy is over-production of inducible nitric oxide synthase (iNOS) within the tumor. We have used recombinant replication defective adenovirus containing the DNA sequences for expression of iNOS in prostate tumor cells and xenograft tumors. The adenovirus gene transfer technology is readily applicable and an iNOS, with CMV promoter, containing adenovirus (Ad5-CMV-iNOS) has been constructed and tested in our laboratory. The technology to inject virus suspension into prostate tumors used herein has been simple injection along a single track with a needle and syringe. This project was aimed at determining the chemical and biological responses of Ad5-CMV-iNOS infected prostate cancer cells growing *in vitro* or as tumors in nude mice. The first phase of this project was aimed at determining the types and quantities of toxic chemical species produced in DU-145 and PC-3 prostate cancer cells in monolayer culture. Emphasis was placed on determining changes in iNOS generation of nitric oxide at low concentrations of oxygen and arginine. Direct cell killing and radiation sensitization was also be determined in Ad5-CMV-iNOS infected cells. In the second phase of the project DU-145 and PC-3 cells were grown as xenograft tumors in nude mice, infected by injection with Ad5-CMV-iNOS, and analyzed for nitric oxide production, cytotoxicity, and radiation sensitization. These experiments were aimed at determining the feasibility of applying iNOS gene therapy to the treatment of prostate cancer in human patients.

5) BODY

The adenovirus vector system (Ad5) used in these experiments produces replication defective virus, as described by Graham (1), and is one of the safest and most practical expression systems for gene therapy. The human iNOS cDNA, as was originally cloned by C. S. Park (2) was inserted into a pΔE1sp1A shuttle plasmid (Microbix Inc.) with a cytomegalovirus enhancer-promoter (CMV) and the SV-40 polyadenylation signal. To produce a replication deficient CMV-iNOS containing adenovirus (Ad5-CMV-iNOS), a co-transfection was performed of the shuttle iNOS pΔA plasmid with the complementation plasmid, pJM17, into 293 cells (transformed human kidney cells that express early region 1 of Ad5). These 293 cells are the host cells and permit successful replication of the Ad5-CMV-iNOS. Production of iNOS-CMV-AD5 vector for use in experiments consisted of infecting 70% confluent cultures of 293 cells with 0.05 MOI iNOS-CMV-AD5 in DMEM:F-12 growth medium with 2% horse serum. For iNOS virus, 50 uM L_NAME was added at the time of infection to block the production of NO while the virus was growing. After about 4 days incubating when signs of cytotoxicity were observed, the cells were removed from the flask surface by agitation, centrifuged, resuspended in growth medium containing 10% glycerol and frozen at –70°C. Later this cell suspension was put through a freeze/thaw procedure alternating 3 times between 37°C and –70°C, to lyse the cells. The large cell debris was removed by centrifugation at 14,000 g. If a high titer virus was need the virus supernatant was purified on a CsCl gradient. The virus fraction was collected, placed in a dialysis cassette(Slide-A-Lyzer, PEIRCE) and dialyzed against two changes of PBS overnight. The viral suspension was then placed in a 100,000 MWCO spin concentrator and the volume was reduced to 10-20% or more depending on the desired concentration. Glycerol was added to the virus to obtain a final concentration of 10% and the adenovirus was aliquotted and stored at –70°C. The viral titer (PFU)was determined using confluent 293 cultures in a plaque assay.

Experiments were performed in which nitric oxide was measured in DU-145 cells exposed to iNOS-Adenovirus at defined MOI and for various durations of expression. Nitric oxide microsensors were prepared as described previously. Briefly, a single sharpened carbon fiber (0.5 to 7.0 μm diameter) encapsulated in a glass capillary with 1 mm protruding (for the extracellular measurements) was modified by coating with nickel(II) tetrakis(3-methoxy-4-hydroxyphenyl)porphyrin by use of cyclic scanning at a potential between -0.2 and 1.0 V. The polymeric porphyrin was subsequently coated by dipping for 5 s in 1% Nation-solution and left to dry in air. Linear calibration curves were constructed for each sensor from $2*10^{-9}$ to $2*10^{-5}$ mol/L NO, before and after measurements, with aliquots of saturated NO standard. The porphyrinic microsensor has a response time of 0.1 ms at micromolar NO concentrations and 10 ms at the detection limit of 1 nmol/L. Platinum wire counter electrode and a silver/silver chloride electrode (SSCE) for reference are placed in contact with measurement media.

For the nitric oxide expression experiments, cells were seeded into 35 mm tissue culture dishes at a concentration of 100,000 cells per dish 24 hours prior to infection. At the designated time after infection, culture medium was changed for serum free medium without L-arginine. Cells were then incubated at 37°C for 1 hour in the L-arginine free medium. Chronoamperometeric measurements (CA) were performed using a PAR model 273 voltammetric analyzer (potentiostat + waveform generator) interfaced with an Pentium II 450 MHz computer with data acquisition and control software. CA, fixed at the peak potential for the oxidation of NO vs SSCE, was used for continuous measurement of the changes of NO concentration from its basal level with time. Because the sensor is located on the surface of cells, and only a very small volume (10 μL) for NO release activation is sampled, the concentration of NO measured is a local or surface concentration (not a bulk or global concentration). Nitric oxide release was activated by

addition of L-arginine to a final concentration of 1 mmol/L in the medium. Results were expressed as nmol/L nitric oxide released after agonist added.

The cells were placed in the controlled environment microscope stage and the nitric oxide electrode moved to near contact with a group of cells. Baseline current was recorded and arginine was added into the culture dish medium to a concentration of 1 mM. Probe measurement was then continued. Arginine stimulation of nitric oxide production was recorded. The first set of experiments was performed to determine the role of time after infection and MOI on nitric oxide release. Viral vector infection was with either iNOS-AD5 clone 7.1 or as a control, β-Gal-AD5. The MOI used were 15 and 75. The results of these experiments are presented in Figures I-1 and I-2. The iNOS-AD5 produced 295 nmol/L NO when virus was at an MOI of 15 and the incubation time at 37°C after infection was 24 hours. The background release rate for non-virus infected control DU-145 cells was 52 nmol/L. Increasing the MOI to 75 for the 24 h time point actually produced a lower NO release of 255 nmol/L. Increasing the incubation time to 48 hours resulted in lower release rates for both 15 and 75 MOI. The rates were 170 and 130 nmol/L respectively. After 72 hours post infection incubation at 37°C the nitric oxide release was similar to that at 48 hours, 150 nmol/L for 15 MOI and 130 nmol/L for 75 MOI. The β-Gal AD5 control vector infected cells actually showed a small amount of stimulation of nitric oxide release, probably due to stimulation of endogenous iNOS. The 24 hour post infection incubation time period had nitric oxide release values of 105 nmol/L and 90 nmol/L for 15 and 75 MOI respectively. The values at 48 hours were 65 nmol/L and 70 nmol/L for 15 and 75 MOI respectively. The values at 72 hours were 80 nmol/L and 85 nmol/L for 15 and 75 MOI respectively. For the iNOS-AD5 virus infected cells substantial cellular toxicity was observed as floating and disintegrating cells. The amount of toxicity increased with both MOI and time of incubation after virus infection.

Figure I-1

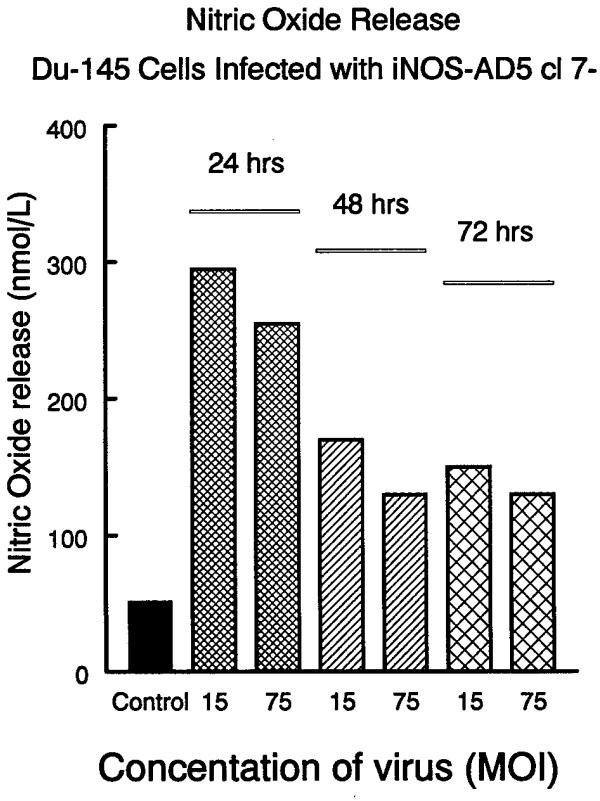
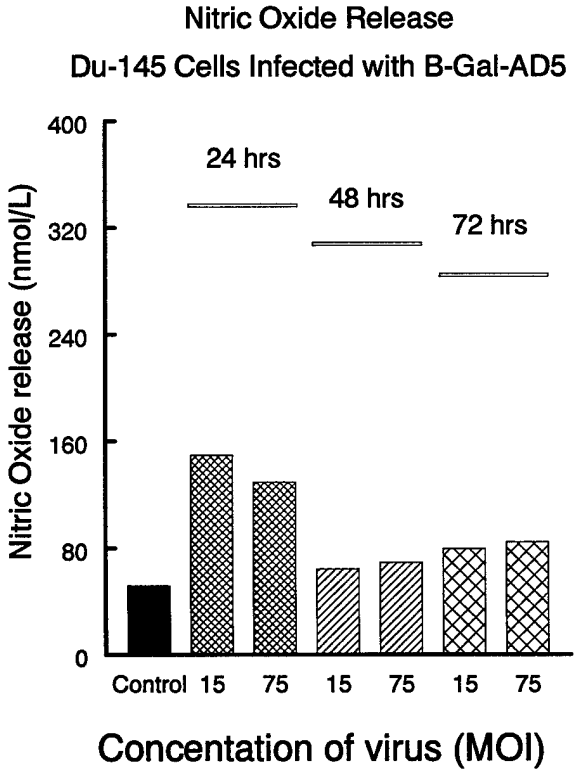
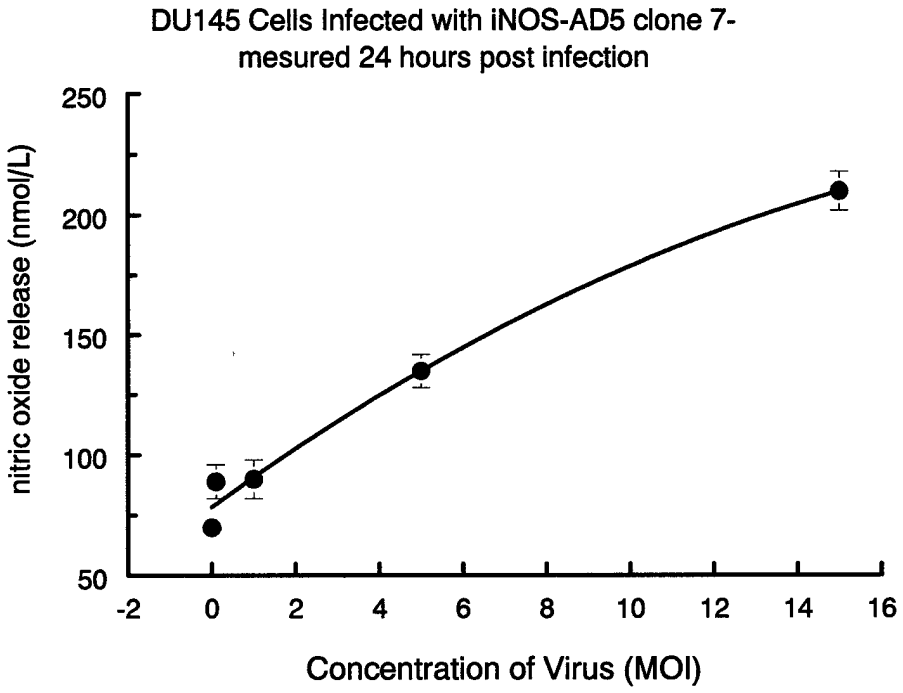


Figure I-2



The next experiments were performed to determine the rate of nitric oxide release at lower MOI of iNOS-AD5 clone 7.1. Because MOI above 15 had produced lower nitric oxide release than that observed at MOI 15, we sought to determine if 15 actually was the peak or if going lower would produce even higher MOI. Cells were grown and infected as described above. The MOI's of iNOS-AD5 used were 0, 0.1, 1, 5, and 15. The results are presented in Figure I-3. The nitric oxide release rate increased with increasing MOI although the curve was flattening at MOI of 15. The release rates for the various MOI were 70 ± 5 nmol/L for MOI 0, 89 ± 7 nmol/L for MOI 0.1, 90 ± 8 nmol/L for MOI 0.5, 135 ± 7 nmol/L for MOI 5, and 210 ± 8 nmol/L for MOI 15.

Figure I-3



The third set of experiments analyzed the role of 37°C incubation time after infection using a wider range of incubation times, especially in the sub 24 hour range. A graphic describing of the experimental protocol is presented in Figure I-4. The results of nitric oxide release rate measurements were presented in Figure I-5. For vector iNOS-AD5 clone 7.1 using MOI 15 the incubation times were 6, 12, 24, 36, and 48 hours. The nitric oxide release rate values were 70 ± 4 nmol/L after 6 hours, 85 ± 3 nmol/L after 12 hours, 152 ± 6 nmol/L after 24 hours, 180 ± 10 nmol/L after 36 hours, and 95 ± 5 nmol/L for 48 hours. A 24 hour post infection point using MOI 30 was also included. The nitric oxide release rate was 50 ± 3 nmol/L for this condition. Another viral vector was also tested. This vector is an AD5 viral clone containing a cDNA sequence of mouse iNOS and the CMV promoter. This vector has been recently developed as described in the next section. The nitric oxide release rate with a 24 hour 37°C incubation after infection for this clone was 190 ± 10 nmol/L.

Figure I-4

iNOS19991001

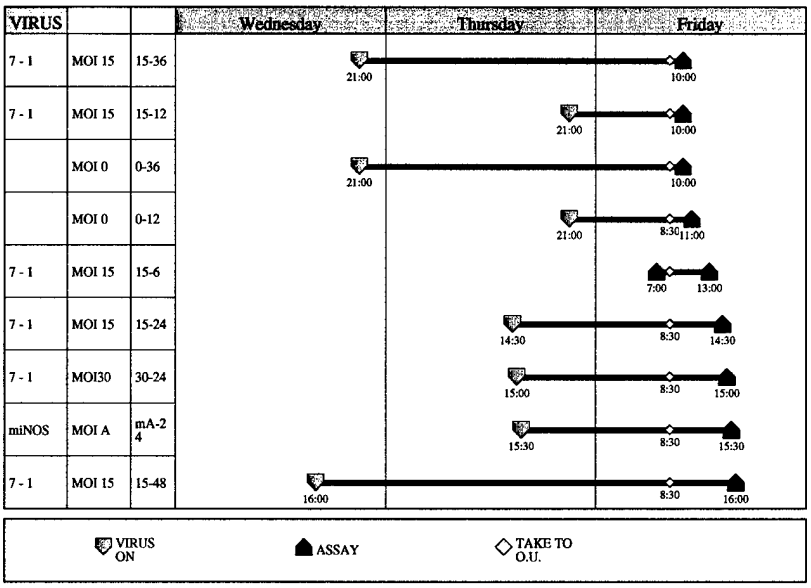
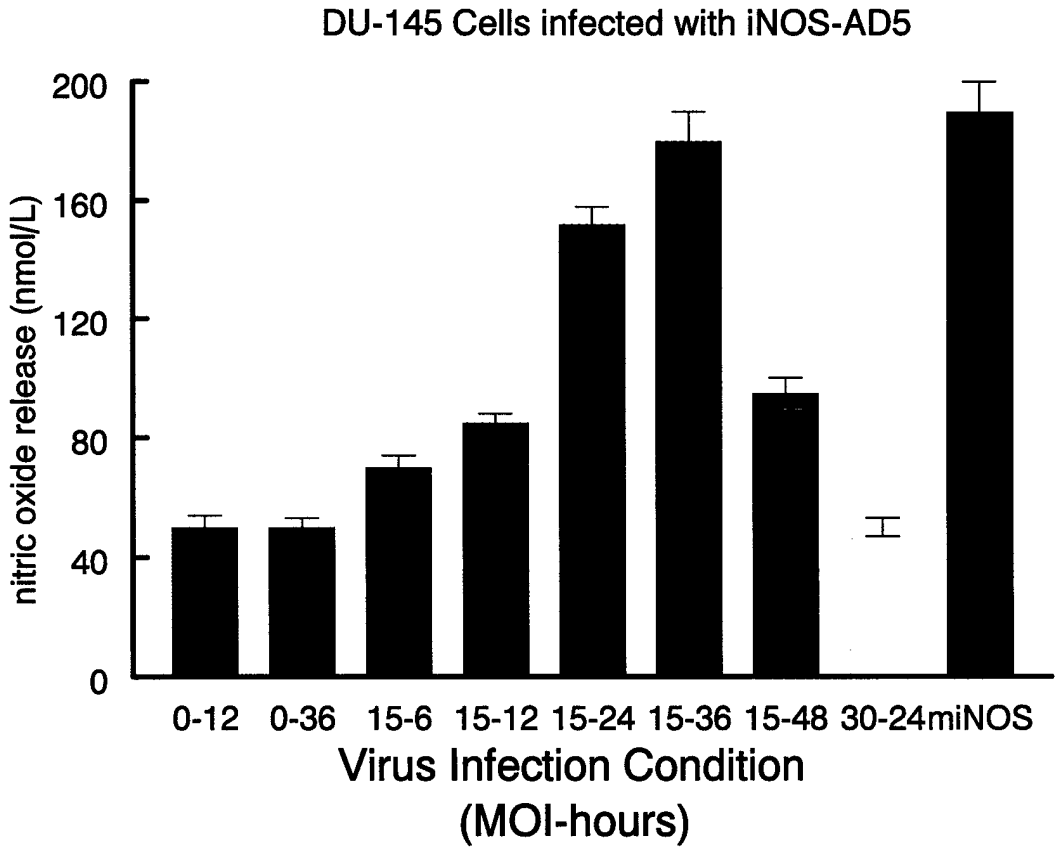


Figure I-5



These experiments have demonstrated that the iNOS-AD5 clone 7.1 vector produces a significant amount of nitric oxide in DU-145 cells and that the time course and MOI must be controlled to result

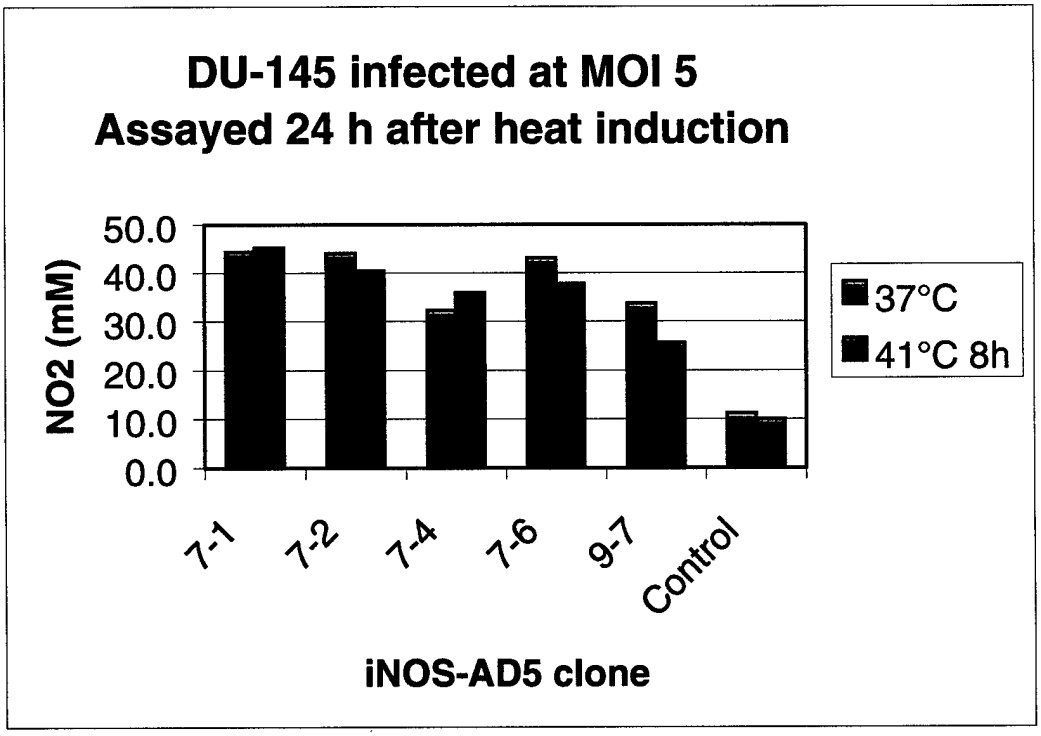
in optimal nitric oxide release. Nitric oxide release increased with increasing MOI up to 15 where after a decrease was observed. The nitric oxide release rate also depended upon time of incubation after infection. The maximum release rate was observed at 24 to 36 hours after infection. Combined with the observed cellular toxicity results we conclude that the decrease in nitric oxide release rate above MOI 15 and time of 36 hours is due to loss of macromolecular synthesis and general cellular metabolism as the cells die. The results also indicate that the new mouse iNOS vector miNOS-AD5 produces NO at least as well as the 7-1 iNOS-AD5 clone.

Do our current "iNOS-AD5" clones have a CMV promoter that is heat inducible?

We learned from our colleague, Dr. Michael Borrelli, that many AD5 virus vectors with a CMV promoter expressed their associated transgene at a much greater rate when infected cells were given a heat shock. The possible benefits of enhancing iNOS expression and thus cell toxicity effects by means of this option interested us enough to do a set of experiments to test whether the "iNOS-AD5" clones have a CMV promoter that is heat inducible. The fact that there are several different forms of the CMV promoter and each behaves differently under various conditions meant that the experimental outcome was uncertain.

DU-145 Cells were incubated in tissue culture dishes for 24 hours prior to infection with iNOS-AD5 viral vector. Five different clones of iNOS-AD5 were used. Several clones were tested because of the possibility of variation in heat enhancement with different clones. Cells were infected at an MOI of 5. After the dishes of cells had incubated at 37°C for twenty four hours after infection, individual dishes were either heat shocked at 41°C for 8 hours or incubated at normal temperature of 37°C for the same length of time. After an additional 16 hours at 37°C an aliquot of medium was removed from the dishes. The medium was assayed for NO2 + NO3 by means of the Griess assay with cadmium conversion of NO3 to NO2. The results presented in Figure I-6 demonstrated that there was no enhancement of NO2 accumulation after the heat shock. The accumulation of NO2 + NO3 in all iNOS-AD5 infected cells was significantly above the control level but no differences between heated and non-heated were observed in any of the five clones.

Figure I-6

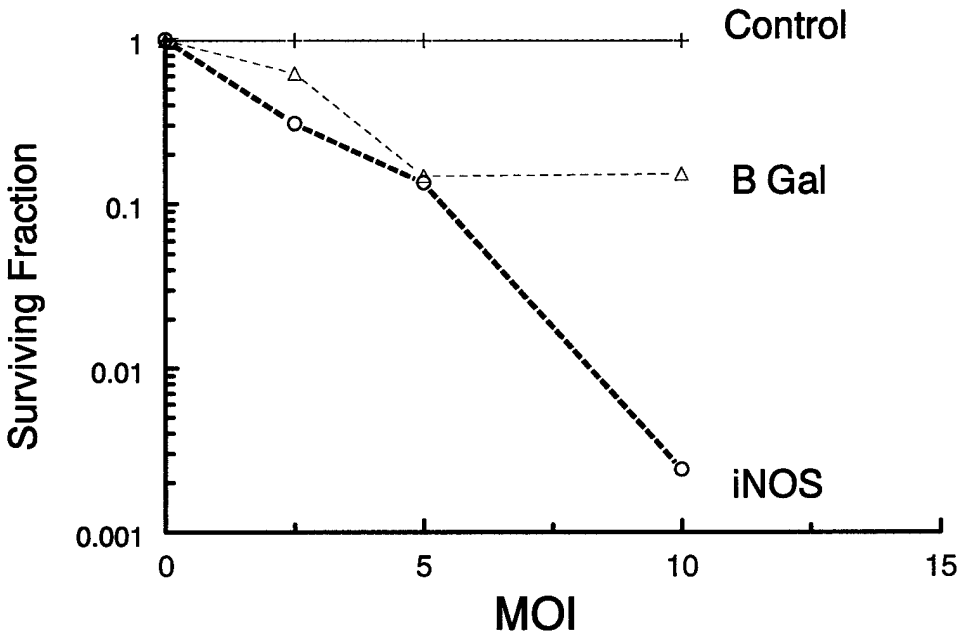


The growth and viability of DU-145 and PC 3 cells was measure after infection with iNOS-AD5 virus vector at various MOI.

Surviving fraction of DU-145 cells was measured using the colony formation assay. Cells were incubated in 60 mm tissue culture dishes for 24 hours prior to infection. Dishes were either infected with no virus, β-Gal-AD5 virus, or iNOS-CMV-AD5 clone 7.1 virus. The MOI's used were 2.5, 5 or 10. Cells were incubated under these conditions for 72 hours. At that point they were removed from the dishes by trypsinization and reseeded into dishes to form colonies. The colony formation dishes were incubated for 10 days prior to staining and counting of colonies. With increasing MOI the β-Gal-AD5 virus infected cells suffered a small decrease in colony formation. The iNOS-AD5 infected cells had only slightly more toxicity than the β-Gal-AD5 infected cells at 2.5 and 5 MOI but at 10 MOI the decrease in survival was much greater with survival almost 2 orders of magnitude lower. The data is presented in Figure II-1.

Figure II-1

Survival of DU-145 cells



Cells were infected with iNOS-AD5 for 72 hours and seeded for colony formation.

GelFoam Survival Assay

A three dimensional tissue culture system was used to analyze the effectiveness of iNOS-CMV-AD5 in sensitizing cells to irradiation. Gelfoam sponge (Gelfoam Dental Pak #4 ;Henry Schein 908-2252), which consists of a collagen matrix, was sliced into 2mm thick by 4 X 7 mm pieces with a microtome. A 25ul droplet of growth medium at a concentration of 1 x 10^4 cells/μl was placed onto an autoclaved Gelfoam piece in a 35mm TC dish and grown at 37°C for 5 days. If the experiment was hypoxic, agar well dishes and a small bottle of medium was placed into the anaerobic chamber incubator. After 24 hr the sponge was moved into the anaerobic chamber and was transferred to the agar well dish with a sterile spatula. (For hypoxic experiments everything was done in the anoxia chamber). After two hours the dishes were transferred to an airtight irradiation box and subsequently irradiated. When irradiation was complete, the sponges with cells were sequentially digested with collagenase F (Sigma C-7926) and of 0.05% trypsin/0.53mM EDTA. Cells thus recovered were seeded for colony formation.

Infecting the sponge with virus

On day-5 the medium was aspirated from the dish and around the sponge. The viral vector (1X10^7 PFU) was added to the top of the sponge in a volume of 10μl. The dish was inverted and incubated for 2 hours at 37°C. The dish was turned over and 2 ml of growth medium was added. The sponges were incubated at 37°C for 24 hr and then treated with radiation as appropriate.

Irradiation

All irradiation was done with a GE Maximar 250 set to 250 KVp and 15 mA. The dose rate in the in vitro irradiation chamber was 1.1 Gy/min. Dose at the irradiation position was measured with TLD. The Gelfoam sponges with cells were placed into an airtight irradiation box which contained the following, in order, from bottom to top:

one warm bag
two activated heat packs
the dish platform
the six agar dishes with sponges centered on the drawn circles (If you don't have 6 use a blank)
the lead sheet with holes positioned over the gelfoam sponges in the wells
the plastic lid with the numbers positioned correctly to match the dishes and appear backward
six nuts tightened with a wrench

The box was placed into the x-ray chamber and cells irradiated with the collimated beam

Digesting the Sponge

When irradiation was complete the sponges were transferred to a 12x75mm snap-cap tube which contained 250ul of ice cold 0.5 mg/ml collagenase F (Sigma C-7926) in DMEM/F12. This was soaked on ice for 15 min. then the tubes put into a rack in the bottom of a 37°C incubator for one minute to warm up, then placed on a warm room shaker for 15 min. The tubes were centrifuged at 1000rpm for 5 min. The medium was carefully aspirated and following was added to the pellet: 250ul of 0.05% trypsin/0.53mM EDTA (the usual trypsin), warm 1 min. in 37°C water and shake in warm room for 10-15 min. (DU = 10 min, PC3 = 15 min.). After trypsin treatment, 250ul of growth media was added and small bored thoroughly. A small amount of cells was removed to count on the hemacytometer. Cells were diluted and plated appropriately.

Experiments

Experiments were performed to determine if hypoxic sparing from radiation killing was observed These results are presented in Figures II-2 and II-3. Other experiments were performed to determine the direct cell killing and radiation sensitization when iNOS-CMV-AD5 was added to the cell containing Gelfoam sponges. Sham treatment, blank, or INOS virus were added 24 hours prior to irradiation and/or seeding. The virus vector condition was 1*10^7 PFU in 10 microliter pipetted onto the sponge as described above. Conditions of normal atmosphere and less than 1 mm Hg hypoxia were used. For hypoxic conditions, the sponge was introduced to anaerobic condition 2 hr before irradiation. Conditions of growth medium without arginine were also tested to analyze the role of low arginine on iNOS toxicity and radiation sensitization.

Figure II-2

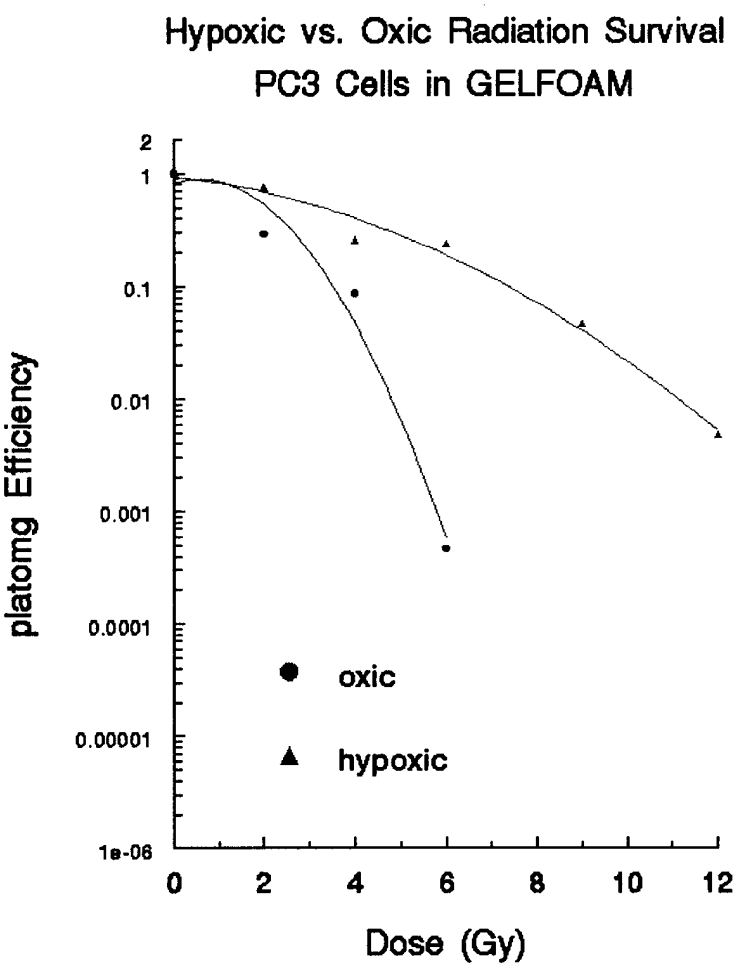
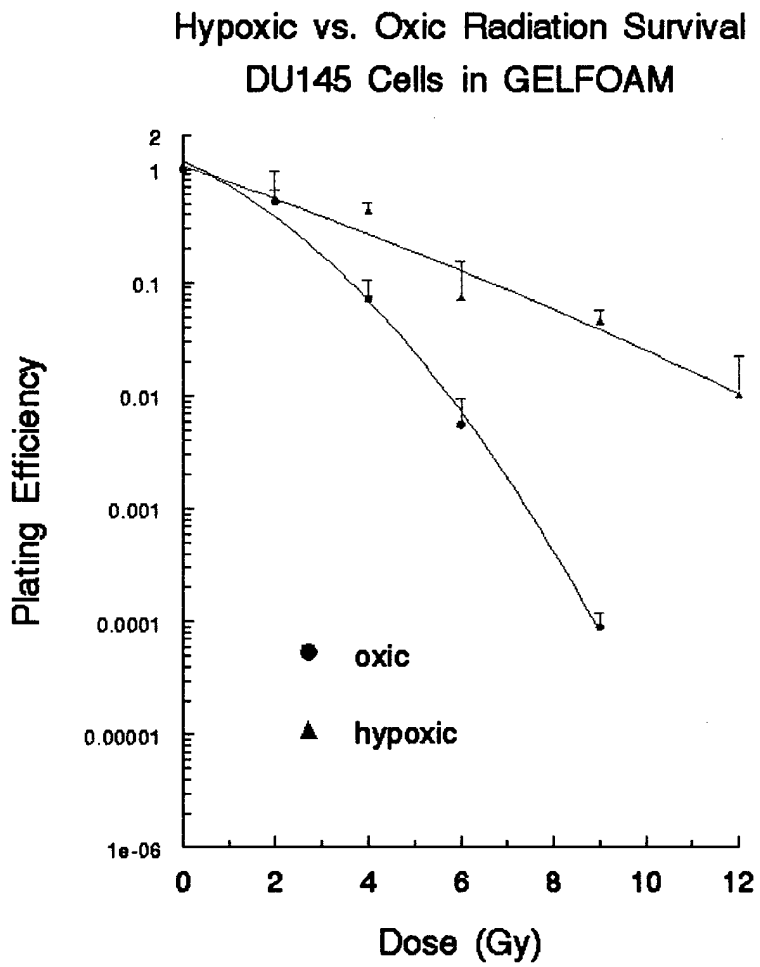


Figure II-3



Cells growing Gelfoam were irradiated either under atmospheric oxygen (Oxic) or anaerobic conditions (Hypoxic) using a custom designed chamber. Du-145 cells had an oxygen enhancement ratio of 2.2 and PC-3 an OER of 2.4.

Figure II-4

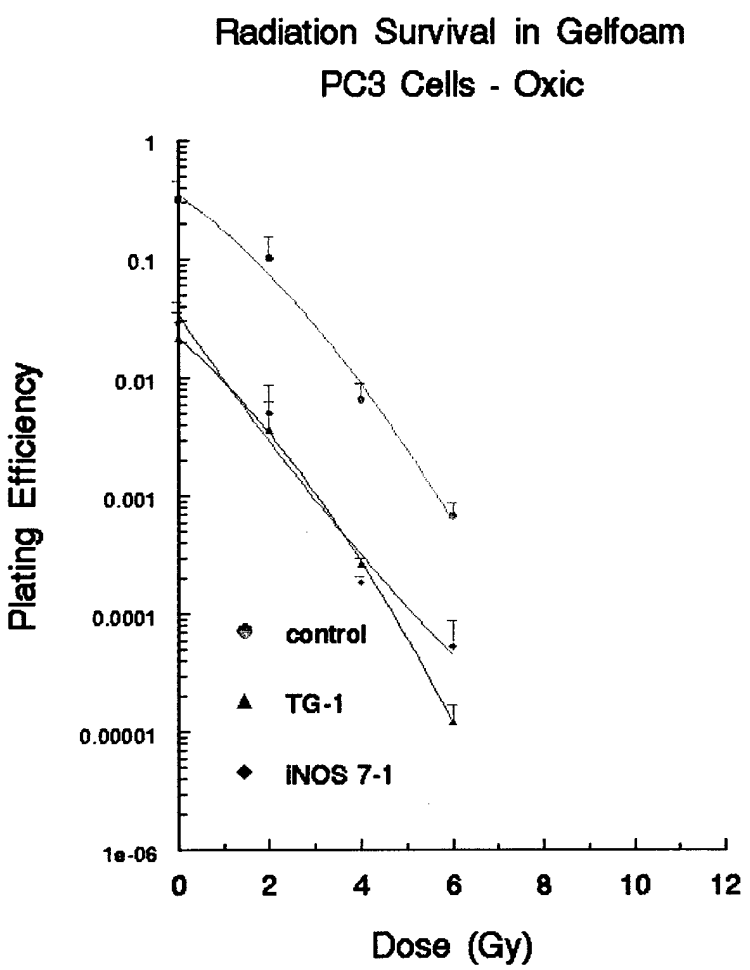
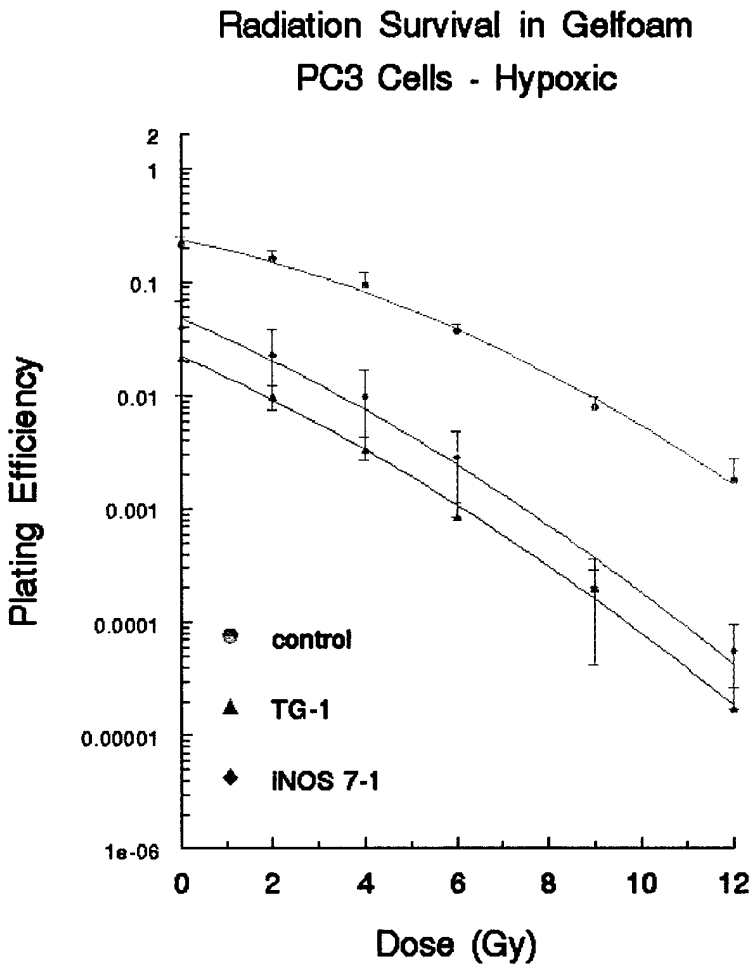


Figure II-5



PC-3 cells were irradiated either under oxic or hypoxic conditions 24 hours after being exposed to sham buffer, a non-cassette containing AD5 vector (TG-1) or the clone 7-1 iNOS-AD5 vector. Both TG-1 and iNOS-AD5 produced direct toxicity under both oxic and hypoxic conditions. No significant sensitization was observed for both TG-1 and iNOS-AD5.

Figure II-6

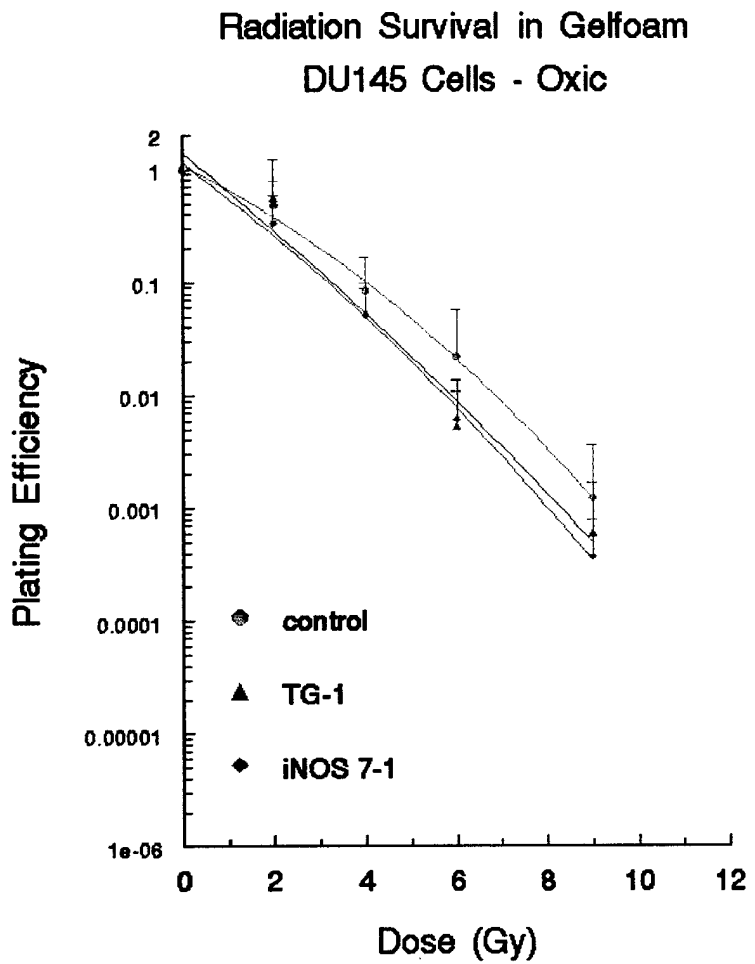
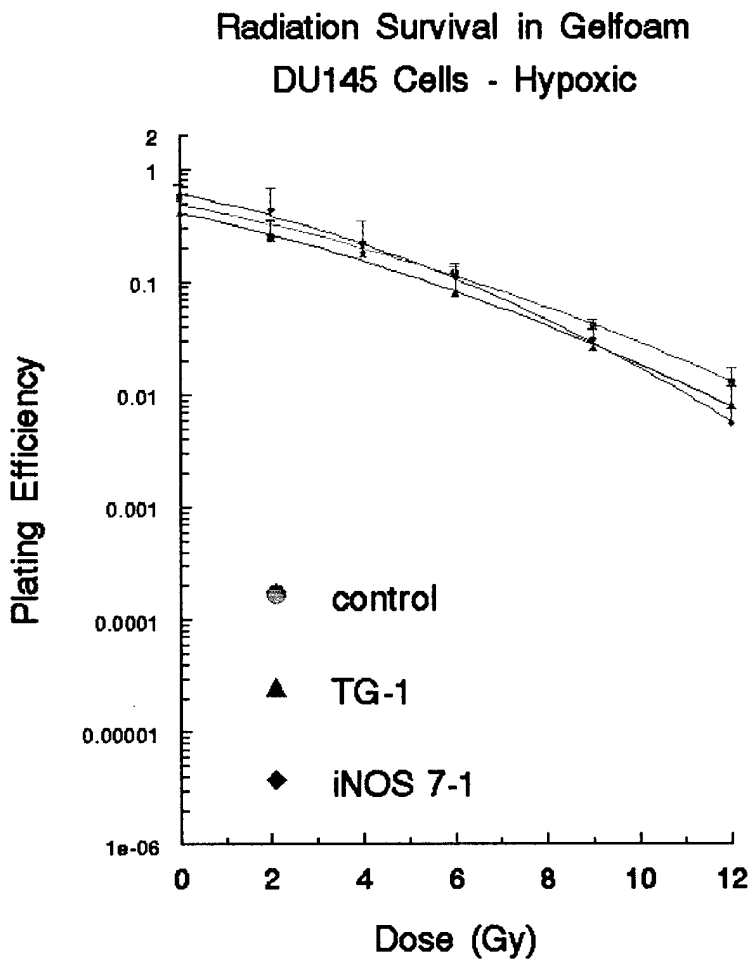


Figure II-7



Du-145 cells were irradiated either under oxic or hypoxic conditions 24 hours after being exposed to sham buffer, a non-cassette containing AD5 vector (TG-1) or the clone 7-1 iNOS-AD5 vector. No effect was observed under hypoxic conditions. Only slight, though non-significant, sensitization was observed under oxic conditions for both TG-1 and iNOS-AD5.

Figure II-8

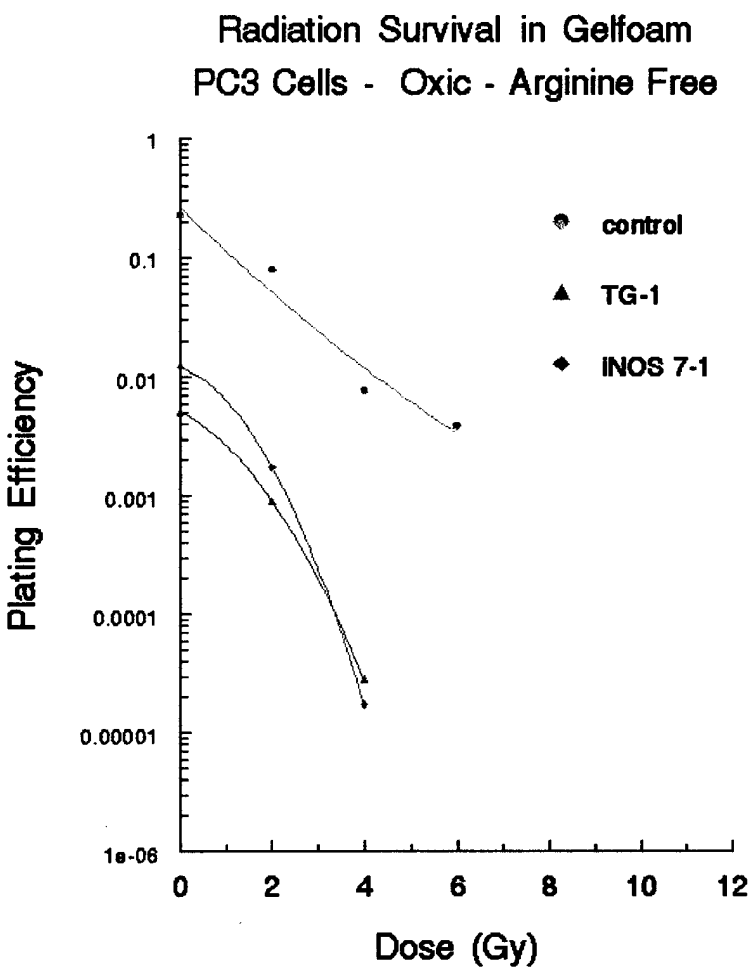
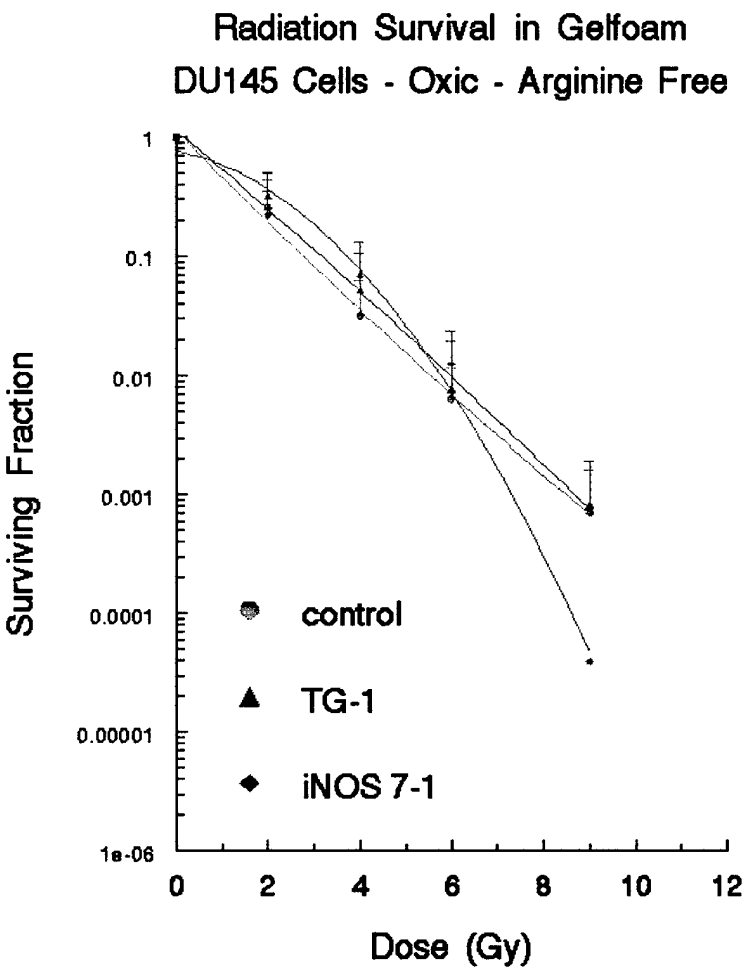
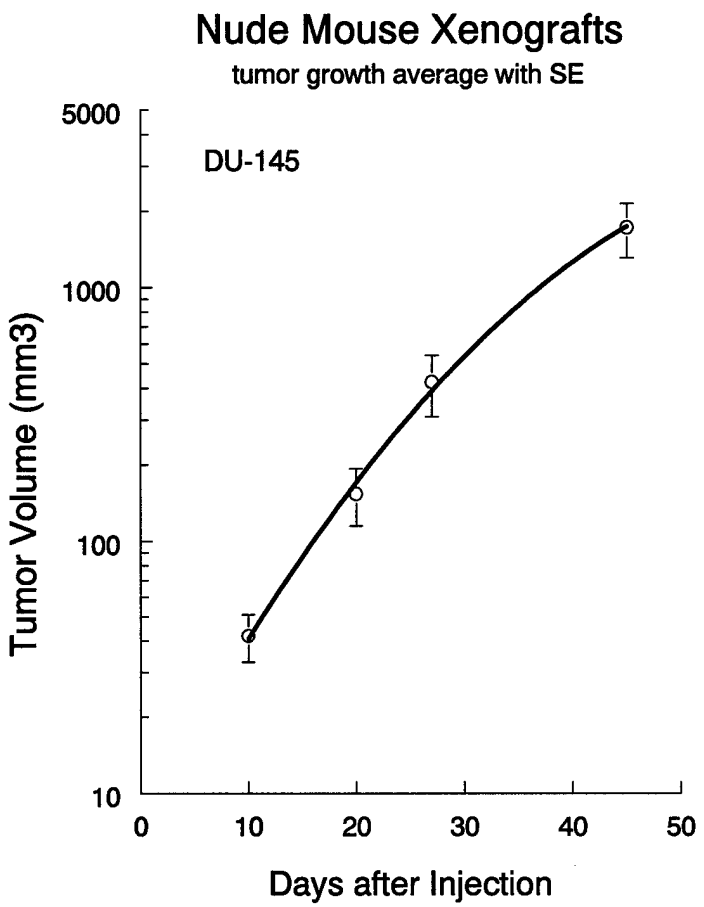


Figure II-9



Du-145 and PC-3 cells were irradiated either under oxic with arginine free medium 24 hours after being exposed to sham buffer, a non-cassette containing AD5 vector (TG-1) or the clone 7-1 iNOS-AD5 vector. Both TG-1 and iNOS-AD5 had survival similar to that with normal medium. No significant sensitization was observed for both TG-1 and iNOS-AD5.

Figure III-1



Initial experiments were performed to establish the growth rates of DU-145 cells growing as xenograft tumors in nude mice under our current conditions, and confirm the tumor cell implant experimental conditions. The growth rate for three tumors averaged is presented in Figure III-1. A small group (12) of mice which were to be the first batch of mice used for a viral infection experiment became sick prior to being entered into the experiment. The problem with the mice was either derived from the vender of the mice or a mechanical failure at the Beaumont Research Services facility. The animal housing conditions were thoroughly reviewed and the problems have not reoccurred.

An OxyLite Photometric fiber optical system (Oxford Optronics, Oxford, England) that is capable of continuously measuring oxygen concentration in tumor or normal tissues for several hours was used in this project to measure oxygen tension in xenograft tumors in nude mice. Two scans through selected mouse tumors were analyzed for hypoxia (oxygen tension < 5mm Hg of O2). Mice were anesthetized prior to measurement, a 20g needle was inserted into the tumor, the OxyLite probe was inserted into the needle and the needle withdrawn, leaving the oxygen sensor in place. The sensor was then moved in increments through the tumor while recording oxygen concentration measurements. The following 4 figures (III-2 to III-5) present oxygen concentration measurement data.

The data demonstrate that both Du-145 and PC-3 tumors have areas of hypoxia in normal breathing mice. Substantial tumor to tumor and intra-tumor spatial variation exists in the distribution of oxygen tension although lowest oxygen levels tended to be in the tumor centers. Values of O2 concentration below 5 mm Hg are considered to be radiobiologically hypoxic.

Figure III-2

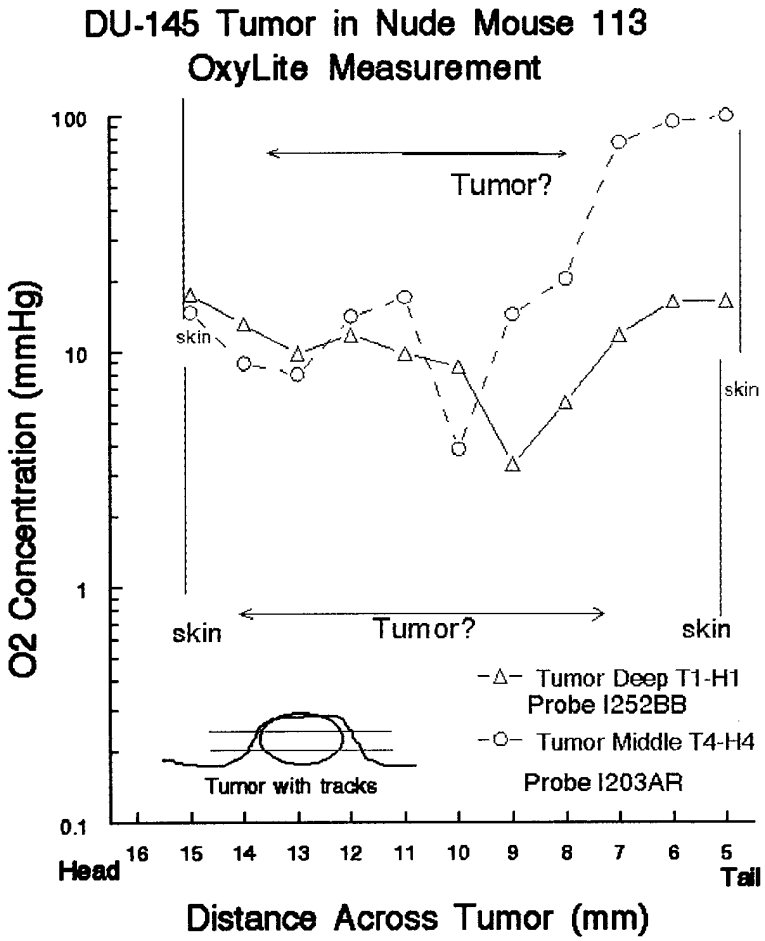
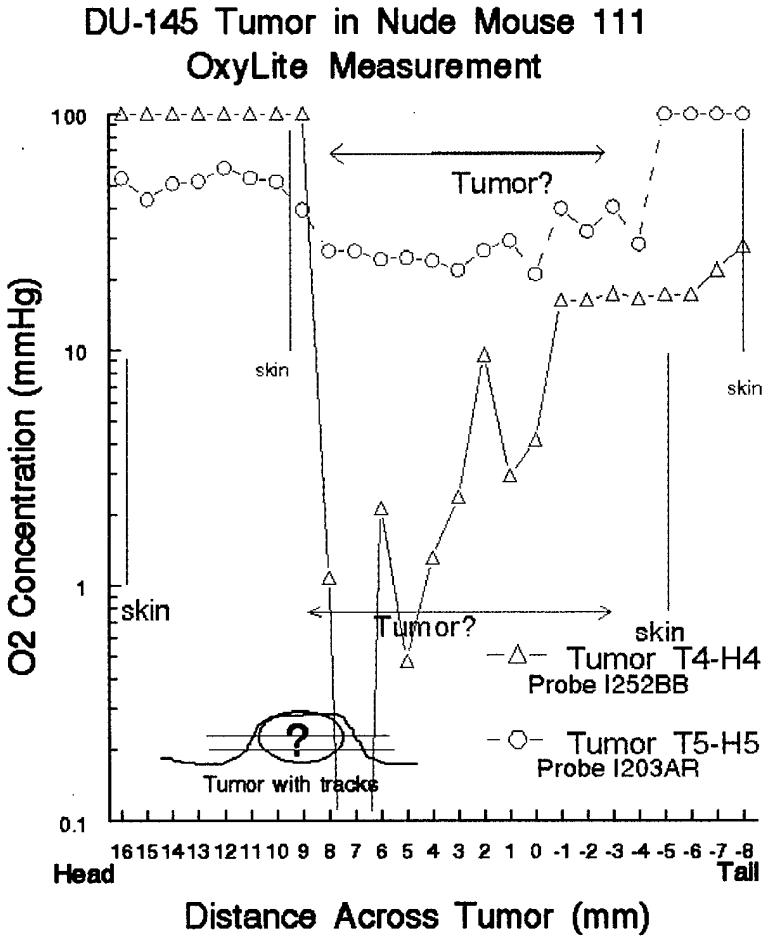


Figure III-3



The O2 concentration was measured along linear tracks in Du-145 tumors growing in the flank of nude mice. The oxygen concentration varied throughout the tumors. Some areas were severly hypoxic (< 2 mm Hg) whereas others were non-hypoxic (>10 mm Hg).

Figure III-4

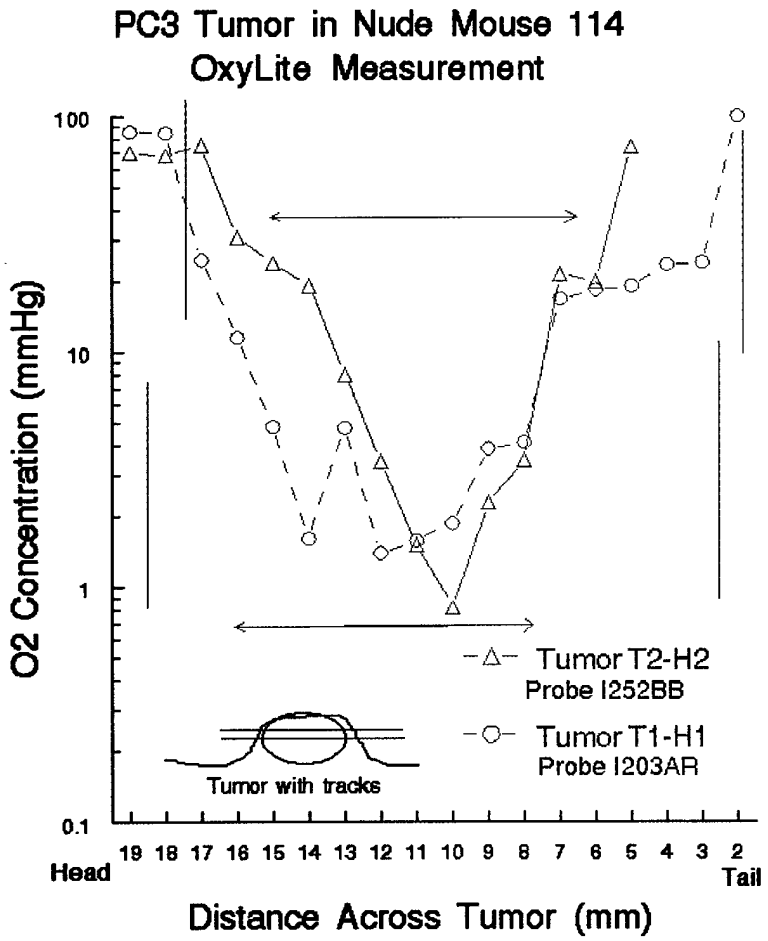
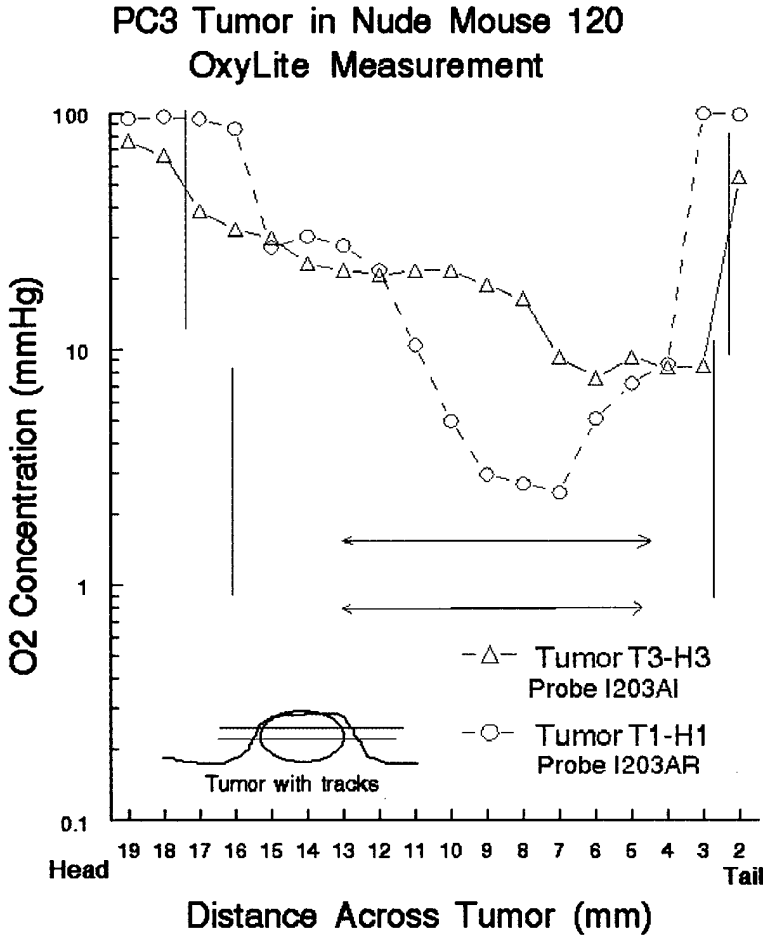


Figure III-5



The O2 concentration was measured along linear tracks in PC-3 tumors growing in the flank of nude mice. The oxygen concentration varied throughout the tumors. Some areas were severly hypoxic (< 2 mm Hg) whereas others were non-hypoxic (>10 mm Hg). These data and others not presented herein indicated that O2 concentration as measured with the Oxylite system was similar in Du-145 and PC-3 tumors. Further measurements in which an oxylite probe was left in the tumor before and after N2 asphyxiation demonstrated that regions of tumors with pO2 greater than 10 mm Hg required 3 to 5 minutes to drop below 5 mm Hg after N2 asphyxiation of the mouse.

In-Vivo/In-Vitro Xenograft Tumor Assay

Tumors were initiated in nude mice by subcutaneous injecting of 2x10^6 cells in 100ul of matrigel into the flank. Tumors reached a volume of about 200 mm^3 at 3 week at which time they were used for experiments. The tumors were injected with 10ul of viral vector suspension at 1x10^9 PFU one day before the experimental treatment irradiation. Normoxic mouse tumors were irradiated with the mouse alive and immobilized using anesthesia. The hypoxic mouse tumors were irradiated 5 minutes after the mouse was sacrificed using general anesthesia and N2 asphyxiation. After irradiation, the tumors were surgically removed with sterile instruments and placed into a preweighed 100mm dish on an ice block. The excised tumor was weighted and the tumor was washed with 5 ml cold serum free medium. The tissue was minced finely (until all pieces were less than a cubic mm). The minced tumor was digested with 200 units/ml collagenase (Sigma C-9407), 100 units/ml hyaluronidase (Sigma H-3884) and 50 units/ml DNAse II (Sigma D-4138) and protease (Sigma P-6141). The live cells were counted on a hemacytometer and seeded for colony formation.

Irradiating the Tumor

Each mouse was anesthetized with 0.025ml ACE /ketamine I.M. If hypoxic the mouse was placed into a chamber purged with Nitrogen gas. All mice were kept warm with 37°C saline bags and heat generation packs. The mouse was loosely taped onto the platform surface in the lucite box. The 250kVp x-ray beam was columinated with ¼" lead shielding such that only the tumor was irradiated. The dose rate to the mouse tumors was 1.9 Gy/min. Dosimetry was measured with a capintec chamber and confirmed with TLD

Removing and mincing the tumor

The mouse was euthanized immediately after irradiation by cervical dislocation followed by bilateral pneumothorax. The tumor was surgically removed with sterile instruments and placed into a preweighed 100mm dish on an ice block. The tumor and dish were weighed. The tumor was washed with 5 ml cold serum free medium which was mostly aspirated from the dish. The tissue was minced as finely as possible (until all pieces are less than a cubic mm) with the dish on the ice block.

Digesting the tumor

The minced tumor was transfered to a 50 ml centrifuge tube containing 2.5 ml of DMEM:F12 (with 15% BCS, VGA, pen/strep/fungizone) and 200 units/ml collagenase (Sigma C-9407), 100 units/ml hyaluronidase (Sigma H-3884) and 50 units/ml DNAse II (Sigma D-4138). The resultant was soaked on ice for 1 hour, then transfer to a 37°C shaking waterbath for 15 min. The digest was spun down at 1000rpm (200xg), the supernatant aspirated and 2.5 ml of DMEM:F12 with 5 mg/ml (6 units/ml) protease (Sigma P-6141) added. Pipeting was done vigorously to break up the tissue pellet and followed by shaking in a 37°C waterbath for 15 min. The tumor digest was pipetted into the syringe and pushed through with the plunger and then passed up and down through the 18g needle about 10 times. The live cells were then counted on a hemacytometer and plated in appropriate growth media supplemented with Penicillin (100units/ml)-Streptomycin (100ug/ml) Gibcoand Fungizone (250ng/ml) Gibco.

Survival curves from the resultant plating efficiencies are graphically presented in the following figures (Figures III-6 to III-9).

Figure III-6

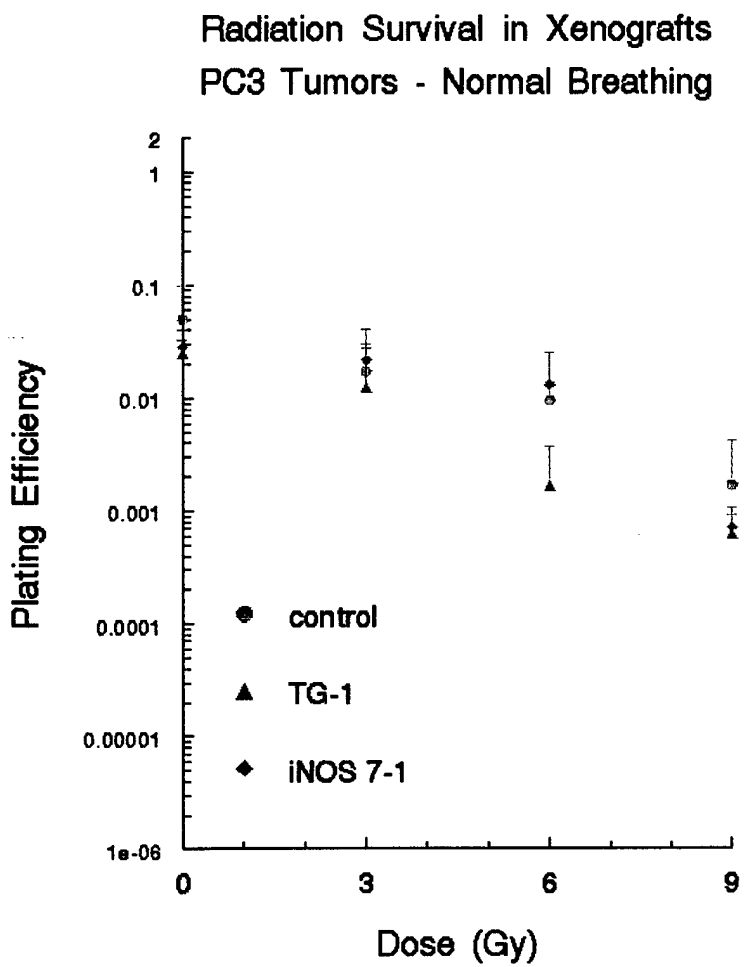


Figure III-7

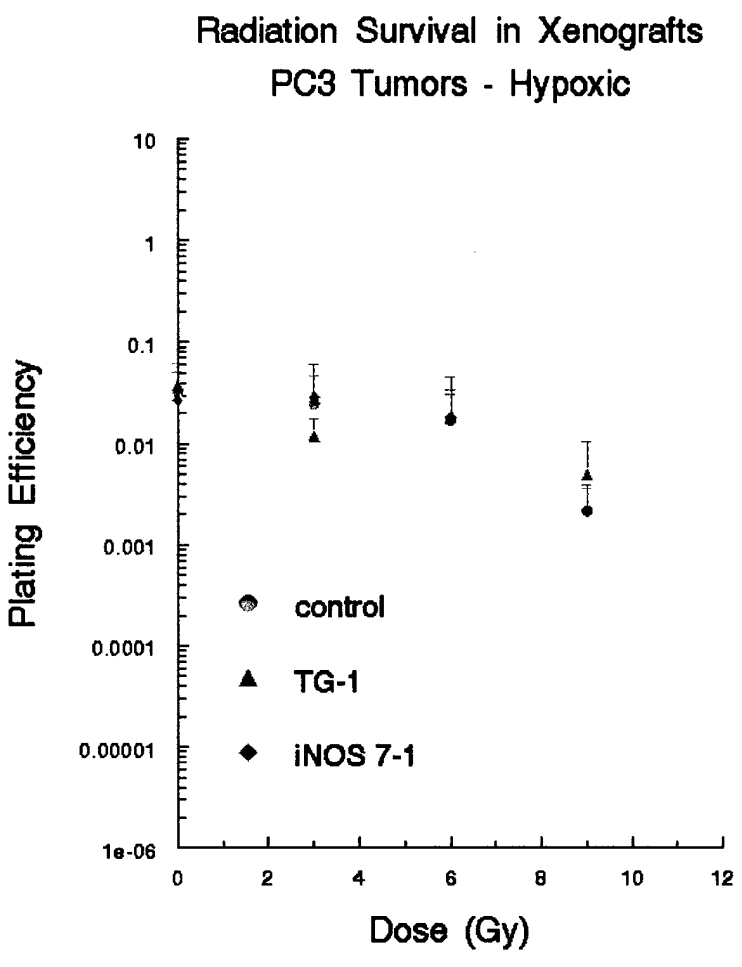


Figure 8 PC-3 cells were grown as xenograft tumors in nude mice to a volume of about 200 mm^3. Tumor were injected with 1x10^9 PFU in 10 µl volume of sham buffer, a non-cassette containing AD5 vector (TG-1), or the clone 7-1 iNOS-AD5 vector 24 hours before being irradiated and assayed by the in vivo/in vitro assay. No significant sensitization was observed for both TG-1 and iNOS-AD5.

Figure III-8

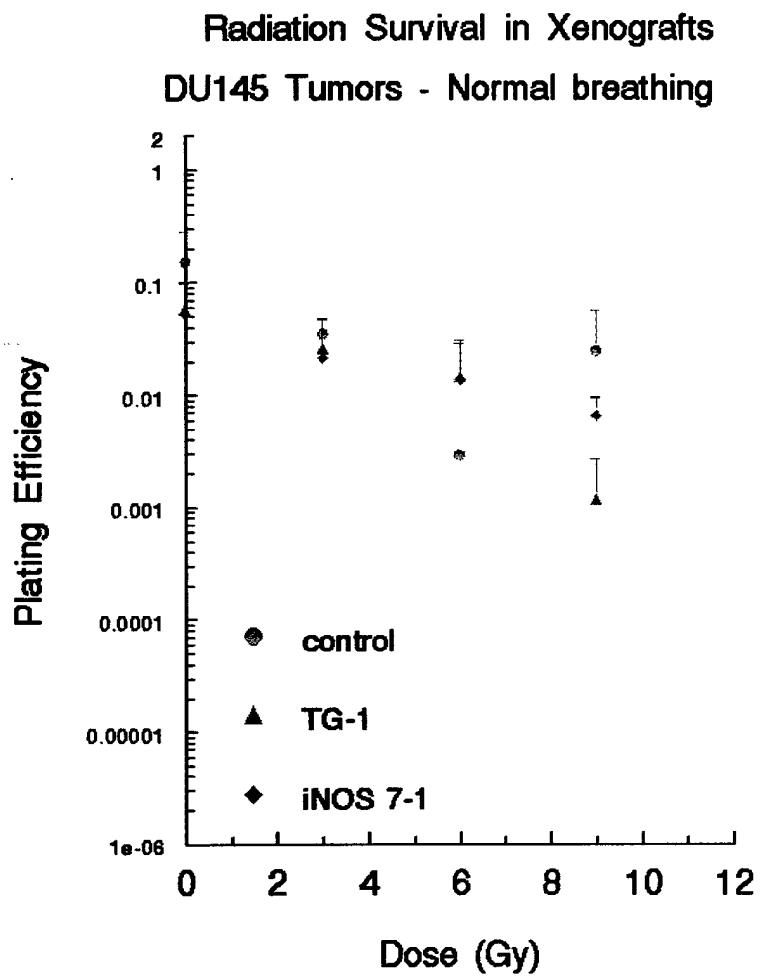
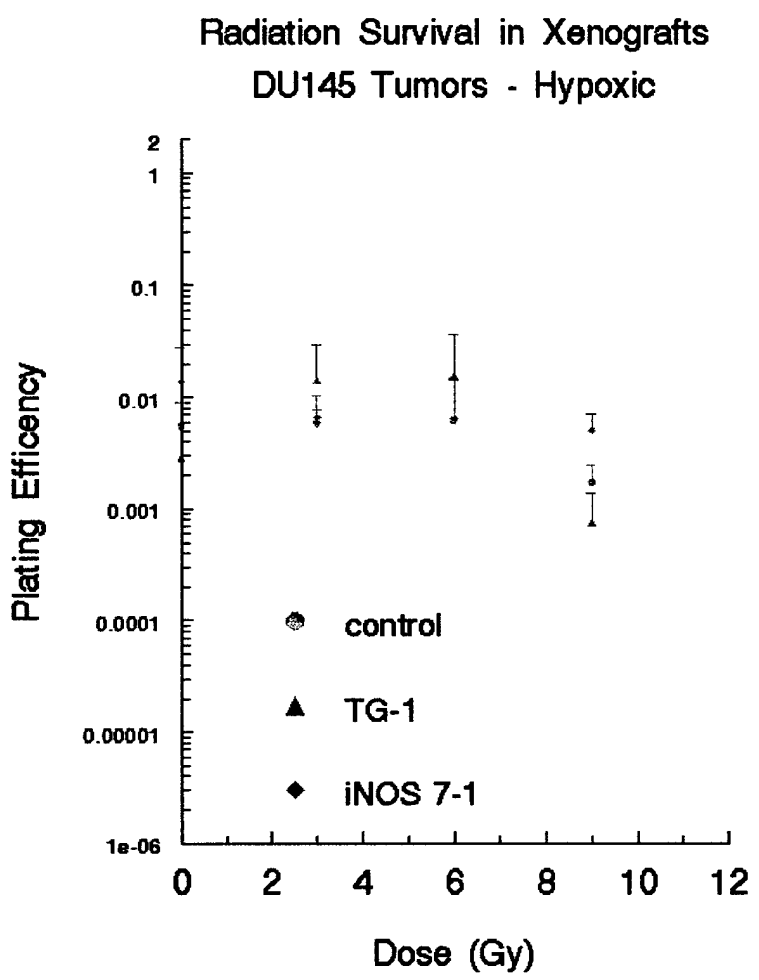


Figure III-9



Du-145 cells were grown as xenograft tumors in nude mice to a volume of about 200 mm^3. Tumor were injected with 1x10^9 PFU in 10 µl volume of sham buffer, a non-cassette containing AD5 vector (TG-1), or the clone 7-1 iNOS-AD5 vector 24 hours before being irradiated and assayed by the in vivo/in vitro assay. No significant sensitization was observed for both TG-1 and iNOS-AD5.

(6) Key Research Accomplishments

- Optimal nitric oxide production by DU-145 prostate cancer cells infected with iNOS-CMV-AD5 clone 7-1 occurred at MOI 15 and in the time range between 24 and 36 hours.
- The CMV promoter on iNOS- CMV-AD5 clones 7-1, 7-3, 7-4, 7-6, and 9-7 effectively promotes production of nitric oxide but is not further induced by heat shock.
- Infection of prostate cancer cells with iNOS-CMV-AD5 clone 7.1 produces direct cytotoxicity as measured by colony formation at MOI greater than 5.
- Observable cytotoxicity after infection with iNOS- CMV-AD5 clone 7.1 at MOI 10 or 20 is initiated as early as day one after infection in DU-145 prostate cancer cells and is severe with partial lysis by day four after infection.
- Large clonal variation must be considered when choosing viral clones for use in expression and cytotoxicity experiments.
- Direct cytotoxicity was observed when PC-3 and Du-145 prostate cancer cells growing in a 3-dimensional collagen matrix were infected with replication deficient iNOS-CMV-AD5.
- No sensitization to acute doses of x-irradiation was observed when PC-3 and Du-145 prostate cancer cells growing in a 3-dimensional collagen matrix were infected with replication deficient iNOS-CMV-AD5 24 hours prior to being irradiated. Lack of sensitization occurred in both aerobic and hypoxic conditions.
- Depletion of arginine from the cell growth medium did not sensitize iNOS-CMV-AD5 infected PC-3 and Du-145 cells under aerobic conditions
- No direct cytotoxicity was observed when PC-3 and Du-145 prostate cancer cells growing as xenograft tumors in nude mice were infected with replication deficient iNOS-CMV-AD5.
- No sensitization to acute doses of x-irradiation was observed when PC-3 and Du-145 prostate cancer cells growing as xenograft tumors in nude mice were infected with replication deficient iNOS-CMV-AD5 24 hours prior to being irradiated. Lack of sensitization occurred in both normal breathing mice and post euthanized tumors.

(7) REPORTABLE OUTCOMES:

- development of cell lines, tissue or serum repositories;

  Several adenovirus vector clones that express the human or mouse iNOS cDNA sequence and produce iNOS protein were developed.

- employment or research opportunities applied for and/or received on experiences/training supported by this award

  A Radiation Oncology medical resident (John Register, M.D.) received research training required for completion of his residency in association with this project. (no financial support was provided to Dr. Register)

  A high school student (Priya Gopwani) received research training for 3 months prior to entering the University of Michigan pre-med program and between her freshman and sophomore years.

  A research grant application submitted and pending funding from the U. S. Army Medical Research Program by P.I. Peter M.Corry, Ph.D. with Co-I Elwood Armour, Ph.D. and Michael Borrelli, Ph.D. was developed with partial support from this project.

- Abstract presented

  Nitric Oxide Gene Therapy for Prostate Cancer
  Elwood P. Armour, Donna McEachern and Tadeusz Malinski
  Radiation Research Society, San Juan, Puerto Rico
  April 20 – 25, 2001

- Publications

  A Collagen Matrix Based 3-Dimensional in vitro Cell System for Radiation and Gene Therapy Research, Elwood Armour and Donna McEachern (in preparation)

  Response of Human Prostate Tumor Cells to iNOS Gene Therapy in a 3-Dimensional Culture System. Elwood Armour, Donna McEachern, and Tadeusz Malinski (in preparation)

- Personnel Paid from Project

Elwood P. Armour, Ph.D.	Principal Investigator
Donna McEachern, M.S.	Research Coordinator
Priya Gopwani	Summer student
Viktor Brovkovych, Ph.D.	Research Assistant (Oakland University)

(8) CONCLUSIONS:

The nitric oxide measurement experiments have demonstrated that the iNOS-AD5 clone 7.1 vector produces a significant amount of nitric oxide in prostate cancer cells and that the time course and MOI must be controlled to result in optimal nitric oxide release. Nitric oxide release increased with increasing MOI up to 15 where after a decrease was observed. The nitric oxide release rate also depended upon time of incubation after infection. The maximum release rate was observed at 24 to 36 hours after infection. Combined with the observed cellular toxicity results we conclude that the decrease in nitric oxide release rate above MOI 15 and time of 36 hours is due to loss of macromolecular synthesis and general cellular metabolism as the cells die. The results also indicate that the new mouse iNOS vector miNOS-AD5 produces NO at least as well as the 7-1 iNOS-AD5 clone. The emphasis in future in vivo experiments will be to focus on events occurring in the 24 to 48 hour time period after infection.

The CMV promoter on iNOS-CMV-AD5 clones 7-1, 7-3, 7-4, 7-6, and 9-7 effectively promoted production of nitric oxide but was not further induced by heat shock. The accumulation of NO2 + NO3 in all iNOS-AD5 infected cells was significantly above the control level but no differences between heated and non-heated were observed in any of the five clones. The CMV promoter will be the promoter of choice in future iNOS adenovirus vector experiments but we will continue to analyze the role of clonal and CMV promoter variation.

Both DU-145 and PC3 prostate cancer cells were observed to be sensitive to being killed directly by infection with iNOS-CMV-AD5 at infection densities as low as 10 MOI. This infectivity level threshold for cytotoxicity correlates well with the level of infectivity that produces increased nitric oxide release. The time course over which iNOS-CMV-AD5 produces cytotoxicity in prostate cancer cells was to be in the range of 1 to 4 days. This rate is more rapid than that observed with radiation and some chemotherapy agents. The relatively rapid loss of cells will be important to managing the consequences of tumor therapy.

Two systems were used to analyze whether iNOS-CMV-AD5 infection could produce direct cell killing or induce radiation sensitization. The systems were 1) 3-dimensional collagen matrix cell system and 2) tumors growing in vivo as xenograft tumors in nude mice. Direct cytotoxicity as measured by the colony formation method was produced by both AD5 containing on insert and iNOS-CMV-AD5. No direct toxicity was observed in xenograft tumors. Radiosensitization was not observed in either the in vitro or in vivo systems. Although hypoxic conditions resulted in significant radiation sparing of PC-3 and Du-145 cells in both 3-dimensional in vitro system and in tumors the oxygen conditions did not affect the role of iNOS-CMV-AD5 in modifying the sensitivity of the cells to radiation. When cells were treated with arginine deficient medium for 24 hours after being infected with iNOS-CMV-AD5 only a slight though not significant sensitization was observed in Du-145 cells.

Unfortunately the overall conclusion of this project is that iNOS-CMV-AD5 vector used herein does not produce significant enhancement of radiation cell killing in Du-145 and PC-3 tumor cells. The reasons for this lack of effect are not obvious but may be related to insufficient NO production due to the chosen promoter. Because the CMV promoter effectively turns on the iNOS expression at all times two problems may have arisen. The first is that the toxic expression of NO during viral vector production may have limited viral clones to inefficient producers of iNOS. We attempted to counteract this possibility by growing 293 cells in the iNOS inhibitor L-NAME during viral generation. We screened many clones for superior NO production but did not find a clearly superior clone. Another possible reason for lack of iNOS effectiveness could be cellular conditioning during the slow build up of NO production due to the continuous expression with the CMV promoter. A controllable promoter such as the heat shock promoter could possibly give a more pulse like expression of iNOS and production of NO. Other techniques that would enhance the absolute expression of iNOS may also be necessary to produce a significant toxicity effect. On the other hand a more mechanistic approach may be needed to determine how iNOS could interact and modify tumor response.

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